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IMMUNOCHROMATOGRAPHIC ASSAY FOR THE FOOT-AND-MOUTH DISEASE UTILIZING RECOMBINANT NONSTRUCTURAL PROTEINS 2C, 3A, 3B AND 3D

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Abstract

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Foot-and-mouth disease (FMD) is a highly contagious infection of domestic and wild cloven-hoofed animals. Recommended measures to prevent transmission of the FMD from endemic areas is a vaccination of livestock in endangered territories and creation of a buffer zone in which susceptible animals are vaccinated. Because FMD vaccines do not provide long protective immunity control for possible infection in vaccinated animals is important in areas with vaccination. It is possible by using DIVA (Discrimination of Infected from Vaccinated Animals) assays which detect antibodies to non-structural proteins (NSPs) of the FMD virus. The paper describes development of an immunochromatographic assay (ICA) which uses recombinantly expressed NSPs 2C, 3A, 3B and 3D as antigens to capture specific antibodies. The recombinant FMD antigens were produced using expression in *E. coli* and purified by metal affinity column chromatography. Sensitivity and specificity of the ICA were found to be 92-97% and 91-96%, respectively in samples of sera from animals with varying risk of the FMD.

Key words: immunochromatographic assay; foot-and-mouth disease; non-structural proteins; antigen; recombinant; diva
List of abbreviations: FMD – foot-and-mouth disease; NSPs – non-structural proteins; ICA – immunochromatographic assay; ELISA – enzyme-linked immunosorbent assay; BSA – bovine serum albumin; RT – room temperature

Introduction

Foot-and-mouth disease (FMD) is a contagious infection in cloven-hoofed animals which is classified as an animal disease of major importance according to the World Organisation for Animal Health (OIE). This disease is endemic in vast regions of the Old World and during outbreaks brings significant economic losses associated with culling of farm animals, implementing quarantine and bearing restrictions on a transportation and trade of livestock and agricultural products.

Selection of the optimal strategy for prevention of the FMD depends on epizootic situation in a particular region. One of the OIE-recommended strategies is a vaccination of all FMD-susceptible livestock in the regions surrounding an endemic area to create a so-called "buffer zone". Kazakhstan approved creation of the buffer zone along borders with neighboring Central-Asian countries which are characterized

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by notoriously high incidence of the FMD outbreaks (Sytnik et al., 2007; Mustafin et al., 2015). The adopted strategy has led to significant improvement in the situation with the FMD exemplified by the fact that during 2014-2015 there were no reported FMD outbreaks in Kazakhstan.

A causative agent of the disease is the foot-and-mouth disease virus (FMDV) which belongs to a genus *Aphthovirus* within a family *Picornaviridae*. The FMDV has a single-stranded RNA genome which encodes virus proteins. Structural proteins constitute virus particle whereas non-structural proteins (NSPs) are not components of virions. Rather the NSPs function for the virus' protein processing and replication machinery. Inactivated whole-virus vaccines predominate for FMD prophylaxis around the world and this is the single type of the FMD vaccines used in Kazakhstan. As this type of vaccines contains killed virus particles it is supposed to elicit antibodies against the FMDV's structural proteins. The NSPs are also immunogenic. Antibodies against the NSPs are important for differentiation of infected animals from vaccinated animals (DIVA) because the infected animals are supposed to develop such antibodies. Kazakhstan farmers use enzyme-linked immunosorbent assay (ELISA)-based DIVA tests such as the SVANOVIR FMDV 3ABC-Ab ruminant ELISA (Svanova) and PrioCHECK FMDV NS Antibody ELISA (Priochek). Both mentioned diagnostic kits utilize the FMDV's antigen 3ABC which is a fusion of immunodominant regions of the NSPs 3A, 3B and 3C. As was demonstrated in a paper of De Diego et al. (1997) presence of antibodies against the 3ABC antigen in sera is a convincing evidence of the FMDV infection. According to this author, all serum samples from infected animals gave positive results in the 3ABC-ELISA regardless of a serotype of the FMDV. In contrast, >99% of sera from the animals vaccinated against the FMD were negative in this test. These results were confirmed by a comparative analysis of three commercial serologic tests: one test was based on a polyprotein 3ABC, the second test utilized natural FMDV antigens and the third test utilized a synthetic NSP 3B (Moonen et al., 2004). Upon adjustment of the cut-off values for each test the best specificity and sensitivity were shown by the test systems based on the 3ABC and 3B.

Utilization of the NSPs in the ELISA allows efficiently identify the virus carriers in infected herds. However, potential hurdles for the DIVA arise when vaccines of low quality are used for the vaccinations. Upon injection with the inactivated FMDV which was not properly purified from a residual material from cells used to grow the virus, anti-NSPs antibodies may appear in vaccines which will compromise the NSP-ELISA results. Quality of the FMD vaccines improved significantly since a beginning of the DIVA testing. Nevertheless, the vaccines containing admixtures of the NSPs are still being used in Kazakhstan which caused an appearance of antibodies to the NSPs in some herds and ambiguous discrimination of vaccinated animals into healthy or convalescent groups. Similar problem was described in Brazil and a particular kind of serologic assays, namely the NSPs-based enzyme linked immunoelectrotransfer blot assay (NSP-EITB) was developed to solve the problem. Diagnostic characteristics of this in-home assay were not inferior to that of the DIVA ELISAs from commercial vendors (Braga et al., 2007). The published NSP-EITB assay employs using of a western blot to test animal sera for reactivity with several NSPs (Neitzart et al., 1991).

This study describes a creation of an immunochromatographic assay (ICA) utilizing the individual NSPs (recombinant 2C, 3A, 3B and 3D) and determining diagnostic characteristics of the ICA.

Materials and Methods

Expression and purification of the recombinant FMDV NSPs

Expression plasmids pET28/2C, pET32/3A, pET32/3B and pET32/3D were transformed into *E. coli* BL21(DE3) electroporation-competent cells (Novagen) using an electroporator. All genetic engineering works were performed using procedures published by Maniatis et al. (1982). Sanger sequencing of the expression plasmids was used to confirm sequences of the genes 2C, 3A, 3B and 3D (Sanger et al., 1977). Portions of 1 ml of overnight cultures were inoculated into 200 ml of Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin and incubated at 37°C with agitation (180 rpm) until OD_{600} reached 0.6. Expression of the recombinant proteins was induced by addition of isopropyl β-d-thiogalactoside (IPTG) to a final concentration of 0.5 mM and incubating at room temperature (RT) for 8 hrs. Bacterial cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C. Cell pellets were resuspended in a binding buffer (20 mM HEPES, 500 mM NaCl, 20 mM imidazole, 5 M Urea, 10 mM 2-mercaptoethanol) and sonicated on ice. Immobilized metal affinity chromatography (IMAC) on the HisTrap HP columns (GE Healthcare) was used to purify the recombinant proteins which have polyhistidine tags on C-termini. Purification was performed in accordance with the manufacturer's instructions. Concentrations of purified proteins were determined by Bradford (1976) and the proteins were analyzed in 12% SDS-PAGE according to Laemmli (1970).

Animal sera

Serum samples from healthy non-vaccinated cattle (n=288) and vaccinated cattle (n=90) were obtained from the Republican Veterinary Laboratory (RVL, Astana, Kazakhstan). Positive

control sera (PCS) (n=25) and a negative control serum (NCS) which are commercially available reference materials which are used in all veterinary laboratories in Kazakhstan were purchased from the LLP "Antigen" (Almaty, Kazakhstan).

Western blot

IMAC-purified recombinant proteins were separated in a 12% SDS-PAGE. Upon electrophoresis proteins were transferred onto a nitrocellulose membrane (Millipore, USA). The membrane was blocked in a phosphate-buffered saline containing 0.1% Tween 20 (PBST) and 1% bovine serum albumin (BSA) and incubated with an anti-His-tag monoclonal antibody (Sigma) as a primary antibody and then with a peroxidase-conjugated anti-mouse IgG (Sigma) as a secondary antibody. Immune complexes were detected by addition of 4-chloro-1-naphthol and hydrogen peroxide.

ELISA

The recombinant proteins were immobilized in wells of a 96-well plate. Aliquots (100 µl) of a solution of the recombinant proteins with a concentration 0.01 mg/ml were dispensed into wells and the plates were incubated for 12 hrs at 4°C. The wells were rinsed and blocked with 1% BSA in PBST. Serum samples from cattle diluted 1:10 in PBST were placed into the wells. Upon 1 hr incubation the wells were thoroughly washed. A solution of the conjugate of anti-bovine IgG with horseradish peroxidase (HRP) (diluted 1:10000 in PBST) was dispensed into the wells and the plate was incubated at 37°C for 1 hr. A substrate solution containing 3,3', 5,5'-tetramethylbenzidine (TMB) was added to the wells. Upon development of color the plate was scanned on a plate spectrophotometer.

Preparation of a conjugate with colloidal gold

Colloidal gold nanoparticles were produced using a hot chloroauric acid-sodium citrate method described by Byzova et al. (2010). The gold nanoparticles were used to produce a conjugate with protein G. Before preparing the conjugate, pH of a colloid solution was adjusted to 6.0 by addition of 0.2 M K_2CO_3. Amount of protein G was measured which provides saturation of surfaces of gold nanoparticles. For this purpose portions (100 µl) of the colloid solution were dispensed into wells of a 96-well ELISA plate. Aliquots (10 µl) of progressive dilutions of protein G were added to the wells and the plate was incubated for 10 min at a room temperature. Upon that aliquots (10 µl) of 10% NaCl were added to each well. Adsorption of the recombinant protein onto the gold nanoparticles results in changes of color of the solution, from red to blue. Optic adsorption in the wells was recorded at 510 nm and 550 nm using a plate spectrophotometer.

Preparation of large amounts of the conjugate was done as follows. A portion (0.2 ml) of a solution of the protein G which contained desired amount of protein G plus a 10% excess was mixed with 10 ml of the colloidal gold (pH 6.0). Mixture was incubated for 10 min at RT and then 0.4 ml of 1% polyethylene glycol (PEG) was added to achieve a final concentration of PEG 0.04%. Upon incubation for 30 min at RT the mixture was centrifuged for 45 min at 60000 g. A supernatant was removed and a pellet was resuspended in 1.5 ml of PBS containing 0.04% PEG as stabilizer. The conjugate was stored at 4°C. Before preparation of immunochromatographic strips the conjugate was diluted 1/10-1/20 in PBS containing 0.02% PEG.

Production of immunochromatographic strips

Methods published by Byzova et al. (2010) were used to produce nitrocellulose membranes with the immobilized recombinant FMDV antigens and to assemble multi-membrane ICA strips. The conjugate of the colloidal gold with protein G was loaded onto a conjugate pad made from a glass fiber matt using an automatic dispenser BioDot XYZ3050. The conjugate was dispensed onto the fiber matt (11 µl per 1 cm). Solutions containing the recombinant proteins (0.5 mg/ml or a different concentration if indicated) and antibodies from normal cow sera (1 mg/ml) were applied onto the nitrocellulose membrane (0.2 ml per 1 cm) upon loading of solutions the glass fiber matt and nitrocellulose membrane left to dry at RT. An absorbent pad, the nitrocellulose membrane, the conjugate pad and a sample pad were assembled to form a complete ICA strip and the latter was assembled into a plastic casing. The complete strips are 65 mm long and 3 mm wide.

Performing ICA

Sample (100 µl) of cattle serum was diluted in 1 ml of a sample dilution buffer (PBST). Sample pad of the ICA strip was submerged in the diluted serum for 2.5-3 min and then the strip was placed on a horizontal surface. Assay results were recorded after 20 min. The ICA result was considered positive if red bands were present in a test line and a control line. The result was negative if a red band appeared at the control line and no coloration was in the test line. If no clear red bands developed on the strip or the only band appeared was in the test line the result was considered invalid.

Results

Four producer strains which express the recombinant NSPs 2C, 3A, 3B and 3D were obtained. The recombinant proteins carrying C-terminal hexahistidine tags were purified using IMAC during which eluates were fractionated. Results of analysis of the fractions in SDS-PAGE showed a presence of proteins

with molecular masses of 40, 45, 38 and 34 kDa (Figure 1). The recombinant proteins 3B and 3D had electrophoretic purity of >90% (Fig. 1C, D). A purification of the recombinant proteins 2C and 3A resulted in an obtaining of fractions with varying purity with the fractions collected at a beginning of the elution containing more admixtures than the fractions at an end of the process (Figure 1 A, B). With the 2C and 3A the purity could be improved by performing a second round of the IMAC. For this purpose the fractions were combined, diluted 1:10 to lower concentrations of imidazole and re-applied to the IMAC column. The recombinant FMDV antigens with purity of no less than 90% were used to construct the ICA tests.

The recombinant proteins were studied using western blots for a presence of a hexahisitidine tags with anti-6-His-tag monoclonal antibodies. Staining with the monoclonal antibodies produces bands corresponding to molecular masses 40, 45, 38, and 34 kDa which are the expected Mw of 2C, 3A, 3B and 3D respectively (Figure 2).

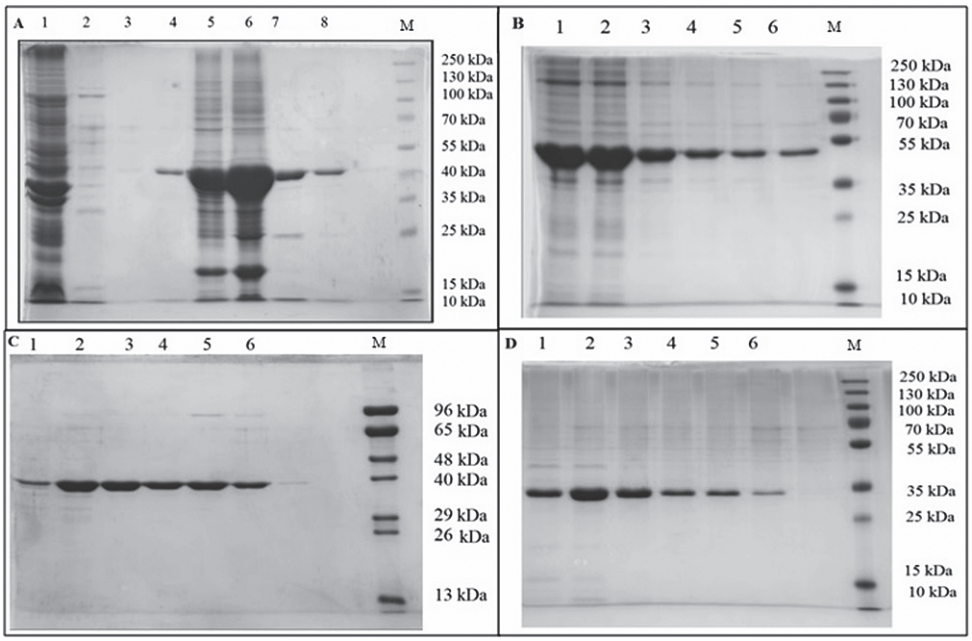

Fig. 1. Analysis of purity of the recombinant NSPs in SDS-PAGE

Panel A, recombinant protein 2C: 1 - flow through, 2 - column wash w/o imidazole, 3,4 - column wash with 50 mM imidazole, 5-8 - eluted protein. Panel B, recombinant protein 3A: 1-6 - eluted protein. Panel C - recombinant protein 3B: 1-6 - eluted protein. Panel D, protein recombinant 3D: 1-6 - eluted protein. M - marker of protein molecular masses

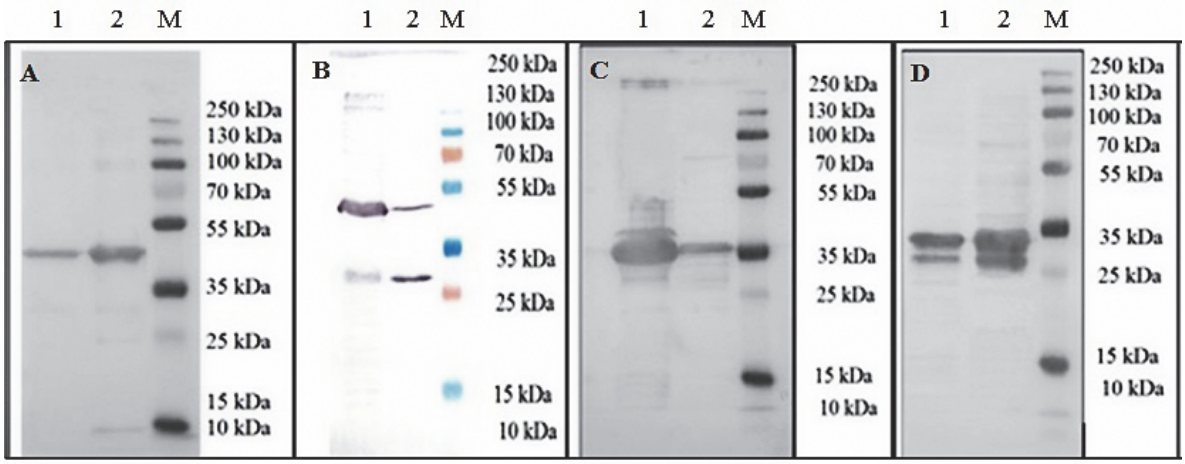

Fig. 2. Western blot of the recombinant NSPs with anti-6His-tag monoclonal antibodies

Panels: A, recombinant protein 2C; B, recombinant protein 3A; C, recombinant protein 3B; D, recombinant protein 3D. Lanes: 1-2, purified recombinant proteins; M, marker of protein molecular masses

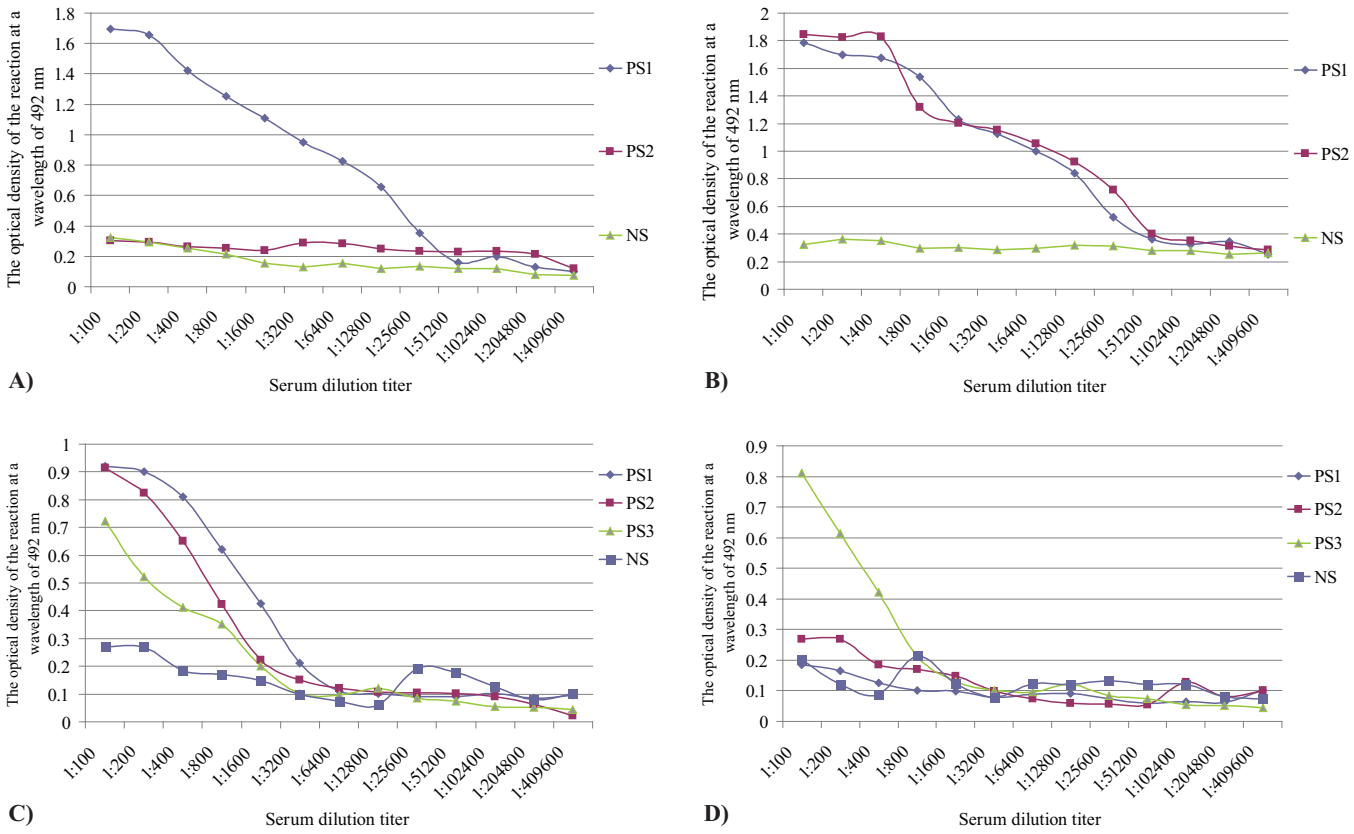

Fig. 3. Indirect ELISAs with the recombinant proteins 2C, 3A, 3B and 3D and dilutions of sera

Panels: A, ELISA with the 2C antigen; B, ELISA with the 3A antigen; C, ELISA with the 3B antigen; D, ELISA with the 3D antigen. Definitions of sera: PS1, antiserum against the FMDV 2C3AB; PS2, antibodies against the FMDV 3ABC; PS3, serum from cows vaccinated against the FMDV; NS, negative control serum from healthy non-vaccinated cow

Ability of the recombinant NSPs to bind specific antibodies in sera from diseased animals is of paramount importance for a use in diagnostic assays. This ability was studied in an indirect ELISA. Initially sera from mice immunized with recombinant FMDV proteins 2C3AB and 3ABC were used for positive controls. Serum from healthy non-vaccinated cows served as negative sera (Figure 3). The recombinant FMDV protein 2C3AB was produced by authors of the paper and will be described elsewhere. Mice were immunized with the 2C3AB (50 μg per mice, mixed with incomplete Freund's adjuvant, intraperitoneally) twice in two weeks. Sera were obtained from the mice on the third week. Commercial antibodies against the FMDV protein 3ABC were purchased from the Absolute Antibody, UK.

A serum from a mouse immunized with the recombinant FMDV antigen 2C3AB reacted with the recombinant 2C antigen in the ELISA in dilutions up to 1:12800 (an optical density at this dilution was 0.623). The NCS and antibodies against the FMDV 3ABC antigen did not react with the 2C antigen. The antiserum against the 2C3AB and the antibodies against the 3ABC reacted with the 3A antigen in dilutions 1:6400 and 1:12800 respectively. Similar results were observed with the 3B antigen. A reaction of antibodies against the 2C3AB and 3ABC were detectable at dilutions 1:6400 in the 3B ELISA. In the 3D ELISA positive reactions were detected only with sera from the vaccinated cows (in dilutions up to 1:1600). Antisera against the 2C3AB, antibodies against the 3ABC and the NCS gave negative results in the 3D ELISA.

To determine a potential of the 2C, 3A, 3B and 3D ELISAs to discriminate sera from cows 288 sera from healthy cows and 25 positive control bovine sera were used (Table 1).

Results from the Table 1 show a high specificity and sensitivity of each ELISA. The specificity varied in a range 91-96% and the sensitivity varied as 92-97%.

We produced a domestic ICA based on the recombinant

Table 1
A specificity and sensitivity of the ELISA

Sera	Qty	FMDV NSPs							
		2C		3A		3B		3D	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Sera from healthy not vaccinated cows	288	13	275	12	276	14	274	24	264
Specificity, %		95		96		95		91	
Positive control sera	25	23	2	24	1	23	2	24	1
Sensitivity, %		92		97		92		97	

Table 2
A comparison of results of the domestic ICA and the IDVet (3ABC) kit

Serum samples	Qty	Domestic ICA								IDVet (3ABC) kit	
		2C		3A		3B		3D			
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Sera from vaccinated cows	90	2	88	4	86	7	83	24	66	26	64
Sera from healthy nonvaccinated cows	100	5	95	4	96	5	95	8	92	4	96
Positive control sera	25	23	2	24	1	23	2	24	1	24	1

FMDV NSPs 2C, 3A, 3B and 3D. A comparison of abilities to discriminate sera from cows with varying FMDV history was done using 90 sera from vaccinated cows, 100 sera from healthy non-vaccinated cows and 25 PCS. The same sera were also tested in the commercial DIVA assay, the 3ABC kit produced by the IDVet. Results are presented in a Table 2.

A study of 90 sera from vaccinated cows showed that the domestic ICAs and IDVet (3ABC) kit revealed differing numbers of positive samples. In this group of sera two samples reacted against the 2C, 3A, 3B and 3D antigens. In addition, two samples from this group contained antibodies against the 3A, 3B and 3D antigens. Importantly, the four mentioned samples were identified as positives in the IDVet (3ABC) kit. From seven samples positive in the IDVet (3ABC) kit three samples were positive in the domestic ICA. From total amount of 24 samples positive in the domestic ICA, 20 samples were found negative by the IDVet (3ABC) kit. These differences can be explained by a use of different antigens: the 3C is present in addition to the 3A and 3B in the IDVet (3ABC) kit and 2C/3D are present in addition to the common antigens in our ICA. In two other groups (the sera from healthy nonvaccinated cows and the PCS) values of a specificity and sensitivity of the ICA and the gold standard (IDVet (3ABC) kit) were comparable.

Discussion

A need to develop an ICA utilizing the separate different NSPs is caused by problems in a differentiation of the FMDV-infected and vaccinated animals (DIVA). In a study of 90 sera from the vaccinated animals the commercial DIVA ELISA kit used as a gold standard revealed 28% positives. Considering a high diagnostic efficiency of this commercial test system based on the 3ABC antigen these data indicate either a use of a low-quality vaccine or clinically silent circulation of the FMDV in the vaccinated herds (Elnekave et al., 2015). However, studies of nasal swabs from cows in these herds by a polymerase chain reaction did not reveal an active infection (data not shown). The main reason for appearance of NSP-antibodies in the vaccinated animals is considered to be the use of low-quality vaccines containing the NSPs as admixtures (Mohapatra et al., 2011). To solve the differentiation problem it is necessary to have in use a diagnostic test system similar to the previously published EITB. In an anticipated test, it be of value to use as antigens the particular NSPs which are absent in the vaccines used for the vaccinations. The main idea of the present work is to develop an ICA utilizing 4 different NSPs, namely the 2C, 3A, 3B and 3D. For a use in the anticipated test the respective recombinant NSPs were produced.

Among promising antigens for a use in the DIVA tests is the 2C protein. Antibodies against the 2C appear during convalescence from the FMD irrespective of a virus' serotype, and the anti-2C are absent in sera from animals vaccinated with vaccines of known high purity (Meyer et al., 1997). The 2C protein is a membrane-bound protein and is not likely to contaminate vaccines. In this study the 2C protein was used among various NSPs to develop an ICA.

Majority of the published DIVA kits utilize the 3A, 3B and 3C antigens. The 3A protein poses a specific interest

because this is the most evolutionary conserved NSP of the FMDV (Paton et al., 2006). A comparison of aminoacid sequences made by the authors of this paper revealed that the 3A from various FMDV strains are very similar (96.7% identity in sequences, data not shown). The 3B protein was also described as a suitable antigen for the DIVA tests (Clavijo et al., 2004; Yakovleva et al., 2006).

As for the 3D protein, it is characterized by a high immunogenicity, so an ICA test based on the 3D antigen is supposed to be suitable to detect the FMD in animals at the earliest stages of an outbreak (Kumar et al., 2012).

The recombinant NSPs 2C, 3A, 3B and 3D demonstrated a valuable use for diagnostics in the ELISA and ICA. The recombinant NSPs demonstrated a high reactivity with antibodies in sera raised by immunizations with different recombinant FMDV NSPs (Figure 2, 3). The DIVA ELISAs utilizing the 2C, 3A, 3B and 3D demonstrated high levels of specificity when 288 sera from healthy non-vaccinated cows were tested: the specificity for the 2C protein was 95%-96%, for the 3A and 3B proteins - 95%, for the 3D protein - 91%. Values of a sensitivity of the ELISAs were tested on 25 PCS; the sensitivity was 92% for the 2C, 97% for the 3A, 92% for the 3B, and 97% for the 3D. These values of the specificity and sensitivity are in good accord with data published in literature (Yakovleva et al., 2006; Mahajan et al., 2013; Mahajan et al., 2015).

In studies of sera from healthy non-vaccinated cows 5-9% of false positives are typically observed. Mahajan et al. (2013) explained these false positives by a nonspecific reactivity. According this author a fraction of anti-2C-positive animals decreased from 14.5% to 1.4% during 180 days after a vaccination. Among samples with persistent anti-NSP reactivity, 1.2% was obtained from herds in areas with a known history of past FMD outbreaks, and thus the reactivity can be attributed to past infections (Mahajan et al., 2013).

Conclusion

Recombinant FMDV NSPs 2C, 3A, 3B and 3D were produced using expression in E.coli and purified using a simple IMAC procedure. The recombinant NSPs reacted in ELISA with specific antibodies in highly diluted antisera. The recombinant NSPs were used to develop an ICA test for a differentiation of infected and vaccinated animals (DIVA). The created ICA showed a diagnostic potential comparable to that of a commercial DIVA ELISA.

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